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Opposite effects of lithium and valproic acid on trophic deprivation-induced GSK-3(Beta) activation, c-Jun expression and neuronal cell death

Ning Jin

Opposite Effects of Lithium and Valproic Acid on Trophic Deprivation-Induced GSK-3β Activation, c-Jun Expression and Neuronal Cell Death

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Index

Abstract

Recent studies demonstrate that lithium and valproic acid (VPA), two commonly used mood-stabilizing drugs, have neuroprotective effects against a variety of insults. Inhibition of the pro-apoptotic enzyme, glycogen synthase kinase-3β (GSK-3β), was suggested to be the mechanism of action of neuroprotection for both drugs. In this study, we tested if lithium and VPA could protect cultured cerebellar granule neurons (CGNs) from GSK-3β-mediated apoptosis induced by trophic withdrawal (serum/potassium deprivation). Lithium concentration-dependently (1–20 mM) protected CGNs. Indirubin, a specific, direct GSK-3β inhibitor, was also neuroprotective. On the contrary, VPA (1-20 mM) did not provide any neuroprotection and even potentiated cell death. Immunoblot analysis revealed that lithium inhibited the trophic deprivation-induced activation of GSK-3β as well as the in vivo phosphorylation of Tau on Ser199, an exclusive target site for GSK-3β. Quite the opposite, VPA did neither inhibit GSK-3β activation nor hinder GSK-3β-mediated Tau phosphorylation. Besides GSK-3β activation, induction of the c-Jun stress response is also essential for apoptosis initiation in trophic deprived CGNs. Therefore we examined the effects of lithium and VPA on c-Jun expression following serum/potassium withdrawal. In accordance with the neuronal survival, lithium prevented the high increase in c-Jun expression, whereas VPA further elevated it. Altogether, our results show that VPA, unlike the common belief, is not a GSK-3 inhibitor and does not provide neuroprotection against GSK-3β-mediated apoptosis.

Introduction

1. Serum and potassium withdrawal of cerebellar granule neurons

During the development of the central nervous system, approximately half of the neurons will undergo apoptosis due to limited amounts of extracellular trophic support, to ensure the formation of a physiologically relevant neuronal network. This death is an important part of the process by which appropriate connections are made between neurons and their targets. This type of cell death (apoptosis) can be mimicked by an *in vitro* system, by using cultured rat cerebellar granule neurons (CGNs) incubated with serum-free and low potassium (5mM) medium. The death of developing neurons in this model has the classic morphological and biochemical characteristics of apoptosis. In addition, mature neurons will undergo apoptosis in response to exogenous toxic stimuli. Such stimuli can be responsible for neurodegenerative diseases. This model system gives us a useful tool to investigate the molecular mechanism of both cell death and the protection from cell death for some specific diseases.

1.1 Inactivation of PI 3-kinase/PKB survival signaling pathway

Recent studies have established a key role for phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (PKB) signaling pathway in regulating trophic factor-dependent neuronal survival. This pathway is essential for neurotrophins rescue of cerebellar granule neurons following serum and potassium withdrawal, and oxidative stress (1). The neurotrophins are a family of structurally related molecules including nerve growth factor (NGF), insulin-like growth factor-1 (IGF-1), and brain-derived neurotrophic factor (BDNF). Under the stimulation of neurotrophins which act through the Trk family of

receptor tyrosine kinases, PI 3-kinase phosphorylates the membrane phospholipid PIP_2 to form PIP_3, leading to the activation of serine/threonine kinase Akt, also known as PKB. Akt/PKB then phosphorylates a number of substrates and modulates the activity of downstream target proteins, which influences multiple cellular functions such as glycogen synthesis, gene expression, and cell survival. One substrate for Akt is Bad, a pro-apototic member that belongs to the Bcl-2 family. Bad induces cell death by stimulating the release of cytochrome *c* from mitochondria. Phosphorylation of Bad by Akt creates a binding site for proteins that sequester Bad in the cytosol, thereby preventing the translocation of Bad to the mitochondrial membrane, thus enhancing the cell survival. Akt may also directly block caspase activation by phosphorylating caspase-9.

In addition to these direct effects on components of the cell death machinery, Akt can regulate GSK-3β activity. Under basal conditions, GSK-3β is constitutively active, phosphorylating and inhibiting glycogen synthase. Once Akt is activated, it phosphorylates GSK-3β on Ser9 and inhibits GSK-3β activity, thus the repression of glycogen synthase is alleviated and glycogen synthesis is favored. The importance of this regulatory action of Akt has gained recognition as multifunctional effects of GSK-3β have been identified on regulating transcription factors, cytoskeletal proteins, and the involvement of cellular events such as embryonic patterning and cell apoptosis. Withdrawal of the depolarizing reagent potassium and certain growth factors contained in serum will lead to inactivation of PI-3 kinase and reduced PKB activity; the chronic depolarization induced by this high concentration of K^+ is thought to imitate endogenous excitatory activity, and low K^+ will potentially decrease the intracellular Ca^{2+}

concentration, which induces apoptosis partially through the suppression of PI3-K activity (2). In turn, the repression on glycogen synthase kinase-3β (GSK-3β) by PKB is relieved and causes dephosphorylation of GSK-3β on Ser9 and hyperactivation. Expression of a dominant-negative (loss of function) mutant of GSK-3β or addition of selective GSK-3β inhibitors can protect CGNs from serum/potassium deprivation-induced apoptosis, suggesting the pro-apoptotic role of GSK-3β in the PI-3 K/PKB pathway negatively regulating neuronal survival (3, 4).

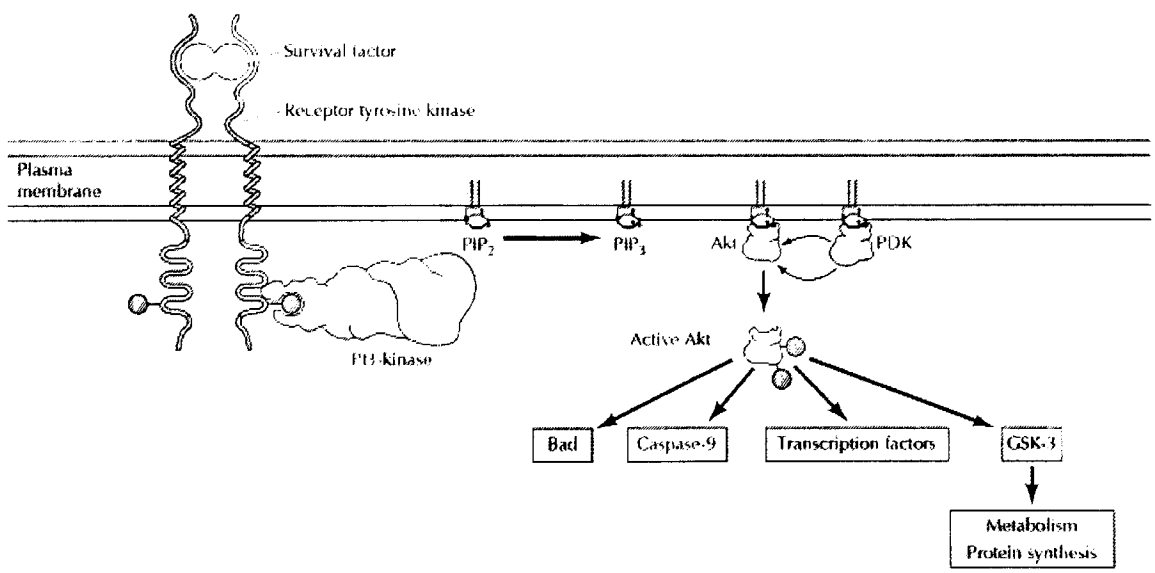


Fig 1. The PI 3-kinase signaling pathway and cell survival. Survival factors such as NGF activate receptor protein-tyrosine kinases, leading to activation of PI 3-kinase and formation of PIP_3. PIP_3 recruits the protein kinase Akt to the plasma membrane where it is activated as a result of phosphorylation by PDK. Akt then appears to phosphorylate a number of proteins that contribute to cell survival. The targets of Akt that have been implicated in suppression of apoptosis include the Bcl-2 family member Bad, caspase-9, several transcription factors, and the protein kinase GSK-3, which affects cell metabolism and protein synthesis (5).

1.2 Activation of stress-associated JNK/c-Jun pathway

Another important cellular event involved in trophic withdrawal-induced cell death is the activation of stress-associated c-Jun N-terminal kinase (JNK) activity, which results in c-Jun protein expression, phosphorylation, and potentiation of c-Jun transcriptional activity. Transcriptionally active c-Jun is a pivotal trigger of apoptosis after neurotoxic insults such as excitotoxicity occurring during ischemia and epilepsy, in response to axotomy and upon withdrawal of trophic support. The activation of c-Jun leads to consequent induction of Bim (a proapoptotic BH3-only Bcl-2 family member), Bax-dependent cytochrome C release into the cytoplasm, caspase activation and cell death (6). Bcl-2 family proteins are important regulators of cytochrome c release. Bcl-2, Bcl-x and Bcl-w are able to suppress apoptosis, whereas Bax is a pro-apoptotic protein that induces cell death. Bcl-2 family members form homo-and heterodimers. The balance between the relative levels of anti- and pro-apoptotic proteins determines cell fate towards death or survival. The anti-apoptotic Bcl-2 family proteins bind to the mitochondrial membrane, whereas Bax in its active form can induce the release of cytochrome c, resulting in the formation of the apoptosome complex consisting of Apaf-1, caspase 9 and ATP (7). Expression of a c-Jun dominant-negative mutant, which cannot be phosphorylated and activated, prevents the death of cerebellar granule neurons following survival signal withdrawal, whereas overexpression of c-Jun induces apoptosis in granule neurons (8). A recent report suggests that GSK-3β may facilitate the downstream events of c-Jun induction, Bim expression and subsequent apoptosis, acting with JNK to coordinate the execution of c-Jun stress response and neuronal death (9).

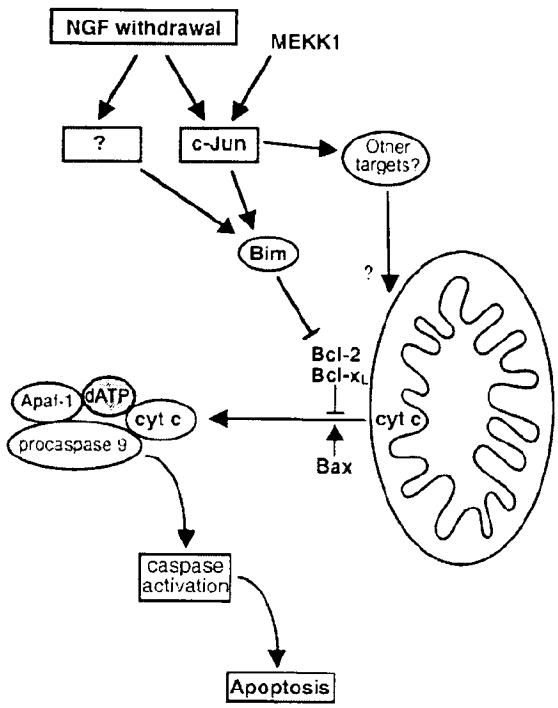


Figure 2. Hypothetical model showing how c-Jun, Bim, and cytochrome c are connected in the dell death pathway in neurons (10).

2. A multifunctional kinase --- GSK-3β

Glycogen synthase kinase-3β (GSK-3β), originally identified as the enzyme capable of phosphorylating and inactivating the enzyme glycogen synthase, is a serine/threonine protein kinase ubiquitously expressed in all eukaryotes. GSK-3β is a key regulator and downstream negative effecter of numerous signaling pathways, including phosphotidylinositol 3-kinase (PI-3k)/protein kinase B (PKB) pathway and Wingless (Wnt) pathway. GSK-3β is involved in a wide range of cellular processes, such as glycogen metabolism, embryonic patterning, cell proliferation, axonal remodeling, and cell apoptosis. Disregulation of GSK-3β activity in the adult brain has been linked to

neuropathology in neurodegenerative diseases such as Alzheimer's disease and certain psychiatric disease. The research on the influence of GSK-3β on neuronal function is tremendous valuable and searching for drugs targeting GSK-3β can be beneficial in exploring the mechanism of how GSK-3β regulates diverse cellular processes.

2.1 Two GSK-3 isoforms

There are two mammalian GSK-3 isoforms encoded by distinct genes: GSK-3α (51kDa) and GSK-3β (47kDa). The difference in size is due to a glycine-rich extension at the N-terminus of GSK-3α. These two isoforms share 98% identity within their kinase domains (11). Homologues of GSK-3 in all eukaryotes examined to date display a high degree of homology. Isoforms from species as distant as flies and humans display > 90% sequence similarity within the kinase domain (12). GSK-3α and GSK-3β, although structurally similar, are not functionally identical.

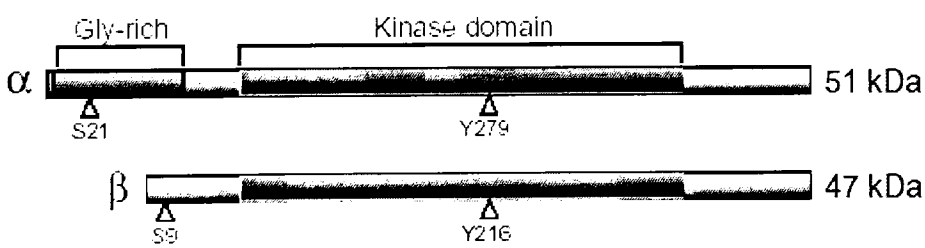


Fig. 3. Schematic representation of mammalian GSK-3α and GSK-3β. Sites of serine and tyrosine phosphorylation are indicated with blue arrowheads. The glycine-rich N-terminal domain unique to GSK-3α and the conserved kinase domain shared by both isoforms are highlighted (13).

2.2 GSK-3β structure and kinase activity

As the crystal structure shows, GSK-3β has the typical two-domain kinase fold with a β-strand domain (residues 25–138) at the N-terminal end and an α-helical domain at the C-

terminal end (residues 139–343) The ATP-binding site is at the interface of the α-helical and β-strand domain and is bordered by the glycine-rich loop and the hinge. The activation loop (residues 200–226) runs along the surface of the substrate-binding groove (14). GSK-3 has a preference for cellular substrates that are primarily phosphorylated, which greatly increases the efficiency of GSK-3β phosphorylation by 100 to 1,000 fold (15). The consensus sequence for GSK-3 substrates is Ser/Thr-X-X-X-Ser/Thr-p, where the first Ser or Thr is the target residue, X is any amino acid, and the last Ser-p or Thr-p is the site of priming phosphorylation (13).

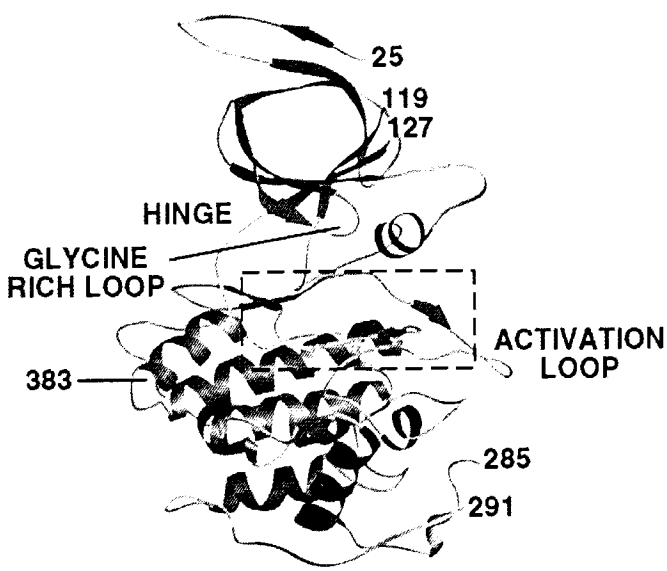


Fig 4. Structure of GSK3β. GSK3β has two-domain kinase fold with a β-strand domain (residues 25–138) at the N-terminal end and an α-helical domain at the C-terminal end (residues 139–343) The ATP-binding site is at the interface of the α-helical and β-strand domain and is bordered by the glycine-rich loop and the hinge. The activation loop (residues 200–226) runs along the surface of the substrate-binding groove. GSK3β has two phosphorylation sites that influence its catalytic activity. Phosphorylation of Ser9 causes inactivation of GSK3. Phosphorylation of Tyr 216, increases its catalytic activity (14).

2.3 GSK-3β phosphorylation and inactivation

Although GSK-3α/β is a constitutively active protein kinase in the resting cell, it is subjected to regulation by numerous enzymes through serine phosphorylation on Ser21 of GSK-3α and Ser9 of GSK-3β. Phosphorylation of Ser9/Ser21 creates a primed pseudo-

substrate that binds intramolecularly to the positively charged pocket and occupies the catalytic site, acting as a competitive inhibitor for true substrate (13).

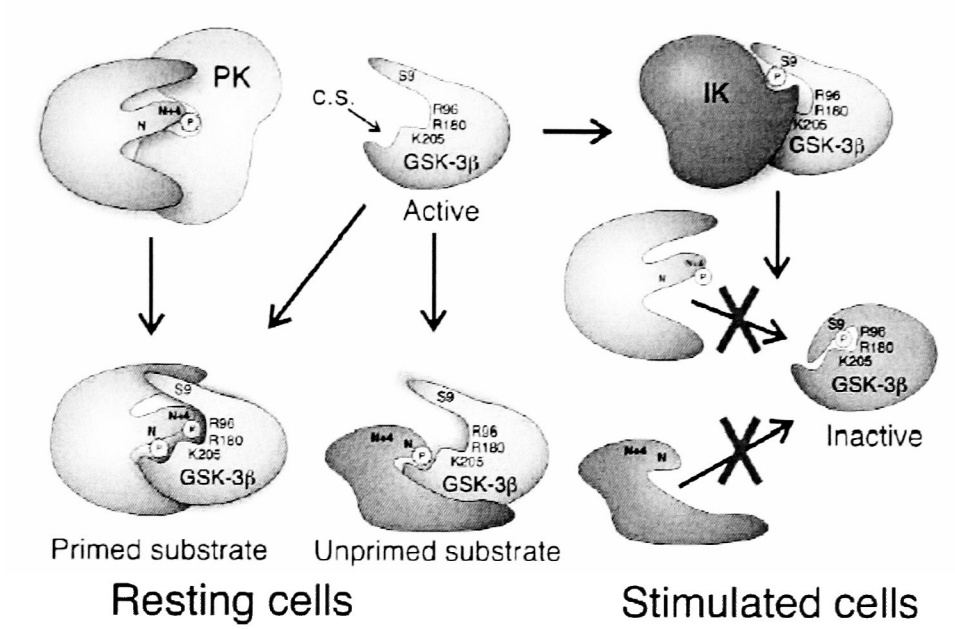


Fig 5. Regulation of GSK-3β activity by serine phosphorylation. Both unprimed substrates and substrates phosphorylated by a priming kinase (PK) are capable of being phosphorylated by the active GSK-3β. The priming phospho-residue at position N + 4, binds a pocket of positive charge arising from the arginine (R) and lysine (K) residues indicated. This directs a serine or threonine at position N to the active catalytic site (C.S.). When an inactivating kinase (IK) such as PKB/Akt phosphorylates GSK-3β on serine 9 (S9), the phosphorylated N terminus becomes a primed pseudo-substrate that occupies the positive binding pocket and active site of the enzyme, acting as a competitive inhibitor for true substrates. This prevents phosphorylation of any substrates (13).

2.4 The role of GSK-3β in PI-3K pathway

As illustrated at the beginning, GSK-3β is negatively regulated by protein kinase B through phosphorylation on Ser9. If PKB activity is repressed under certain conditions, such as serum deprivation or potassium withdrawal, GSK-3β can be dephosphorylated, indicating its hyperactivation.

2.5 The role of GSK-3β in *Wnt* pathway

The *Wnt* signaling pathway, named for its most upstream ligands, the Wnts, has been demonstrated to play important roles in controlling embryonic patterning, cell fate, cell proliferation, and differentiation. Its role in cell development has been probed in fruit flies, nematodes, zebra fish, frogs, and mice. Many of the known *Wnt* loss-of-function mutations have been generated in the mouse and some highly intriguing phenotypes are seen. For example, inactivation of *Wnt*-7a results in animals with dorsal-ventral polarity limbs (16). Similarly, the phenotype of *Wnt*-4 mutations causes kidney defects (17).

Members of the *Wnt* family interact with trans-membrane receptors of the Frizzled family. The binding of *Wnt* by the Frizzled protein causes the Frizzled protein to activate the Disheveled protein. Once the Disheveled protein is activated, it inhibits the activity of GSK-3β. If GSK-3β was active, it would phosphorylate the N-terminal domain of β-catenin, thereby targeting it for ubiquitylation and proteosomal degradation. However, when the Wnt signal is given and GSK-3β is inhibited, β-catenin is dephosphorylated, accumulated in the cytosol and then translocated into the nucleus. Once inside the nucleus, it can form a heterodimer with an *LEF/TCF* DNA-binding protein, becoming a transcription factor. This complex binds to and activates the Wnt-responsive genes (18). Mutations in β-catenin that prevent its phosphorylation by GSK-3β have been found in cancers of the skin, colon, prostate, liver, endometrium and ovary (19).

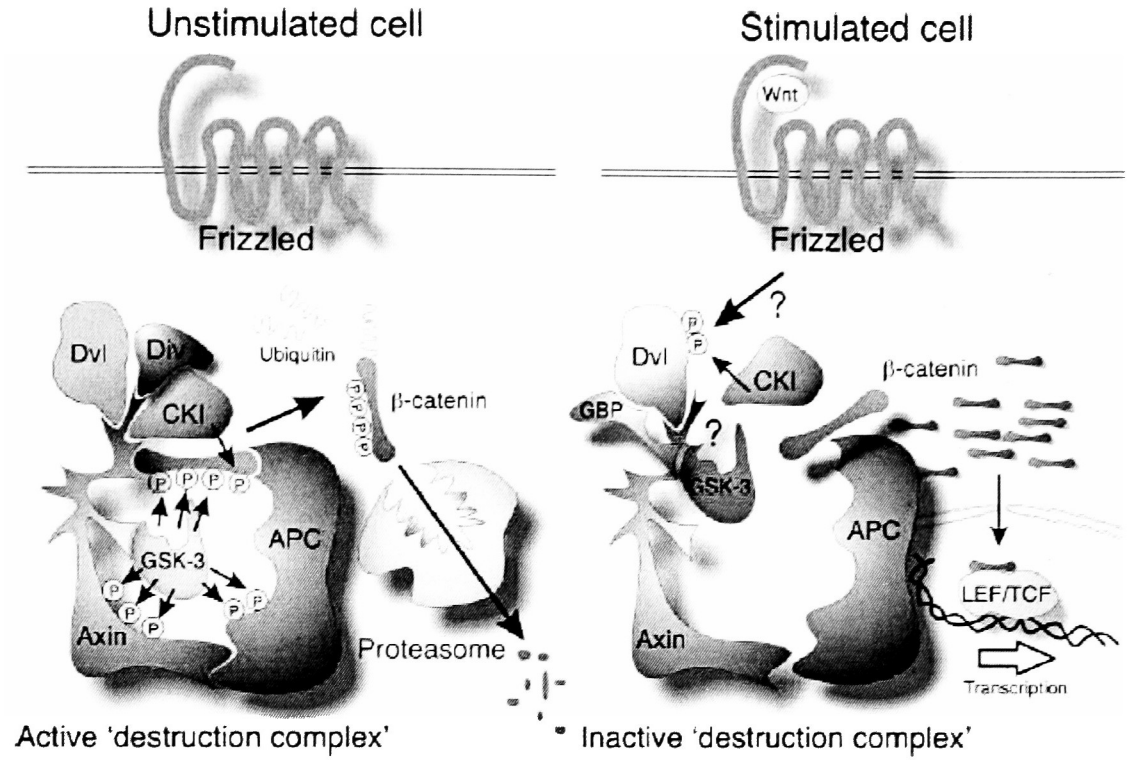


Fig 6. The Wingless pathway Wingless (Wnt) polypeptides bind to cell surface receptors of the Frizzled family. Signaling from Frizzled leads to inhibition of the protein kinase GSK-3, resulting in stabilization of β-catenin and an increase in the concentration of β-catenin in the cytosol. Free β-catenin then forms a complex with the transcription factor LEF1 and the β-catenin/LEF1 complex translocates to the nucleus and drives gene expression (13).

2.6 GSK-3β and axonal remodeling

As the above illustrates, Wnt factors regulate cell fate decisions in early development. Moreover, WNTs also play important roles later in the development of the central nervous system by regulating the axonal cytoskeleton and the clustering of synaptic proteins at nerve terminals (20, 21). WNT-7a can increase the growth cone size and axonal spreading along the axon in developing neurons. In addition, WNT-7a increases the clustering and level of synapsin I, a presynaptic protein involved in synapse formation

and function (22, 23). Also, lithium, a direct GSK-3β inhibitor, can mimic the effect of Wnt-7a on the axonal remodeling and synaptic protein clustering. This suggests that GSK-3β may play an important role in axonal remodeling and presynaptic differentiation (20).

2.7 Other GSK-3β substrates

GSK-3β phosphorylates numerous proteins including transcription factors, translation factor and other proteins involved in cytoskeletal regulation, intracellular vesicular transport, cell cycle progression, circadian rhythm regulation and apoptosis. Phosphorylation of these substrates by GSK-3β is most often inhibitory, as in the case of glycogen synthase, β-catenin (13). Microtubule-associated protein (MAP) tau, the structural protein that promotes tubulin polymerization and stabilizes microtubules, is a well–established physiological substrate of GSK-3β. Hyperphosphorylated tau is the primary component of neurofibillary tangles (NFTs), which is one of the neuropathological hallmarks of Alzheimer's disease. Tau phosphorylation involves numerous kinases, such as GSK-3β, cyclin-dependent kinase 5(Cdk5), P38 kinase, and c-Jun N-terminal kinase (JNK). Among the abnormal phosphorylation sites identified, GSK-3β specifically phosphorylates serine 199, 202, 235, 396, 404 and 413, and threonines 205 and 231 (24).

2.8 GSK-3β and Alzheimer's disease

Alzheimer's disease (AD), the most common neurodegenerative disease, is characterized by progressive memory loss and impairments in language and behavior that ultimately

lead to death. The cognitive decline in AD is accompanied by neuronal atrophy and loss, mainly in the cortex, hippocampus and amygdala. In addition to a specific pattern of neuronal cell death, AD is characterized by two neuropathlogical hallmarks, senile plaques and neurofibrillary tangles (NFTs). Senile plaques are extracellular deposits of amyloid fibrils composed of the 39–43 amino acid β-amyloid peptide (Aβ) often surrounded by dystrophic neuritis. NFTs are intraneuronally generated aggregates of paired helical filaments (PHFs), which are assembled from the hyperphosphorylated form of tau (25).

GSK-3β has been linked to many of the major neuropathological mechanisms that are associated with Alzheimer's disease. GSK-3β was found to be identical to tau protein kinase I (TPK I), an enzyme that was identified in brain extracts due to its ability to phosphorylate tau in vitro. GSK-3β is one of the best candidates for generating the hyperphosphorylated tau. It has been shown to phosphorylate tau in most sites that are found in PHFs, both in transfected cells and *in vivo*. Furthermore, GSK-3β accumulates in the cytoplasm of pretangle neurons, and its distribution in the brains staged for AD neurofibrillary changes is coincident with the sequence of development of these changes (25).

It is reported that GSK-3β phosphorylates the amyloid precursor protein (APP). The exposure of cultured cortical and hippocampal primary neurons to neurotoxic β-amyloid (Aβ) derived from cleavage of the APP can induce activation of GSK-3β (26), tau hyperphosphorylation (27) and cell death (27, 28). Blockade of GSK-3β expression or activity, either by antisense oligonucleotides or by lithium (a direct GSK-3β inhibitor), prevents Aβ- induced neurodegeneration of cortical and hippocampal primary cultures

(28, 29). Furthermore, transgenic mice conditionally over-expressing GSK-3β display hyperphosphorylated tau and some signs of neuronal degeneration (25).

In addition, GSK-3β interacts with presenilin proteins, encoded by the genes (PS-1 and PS-2) that are responsible for triggering inherited forms of AD (familial AD). Several investigators recently reported that presenilin 1 binds to GSK-3β (30), and facilitates the co-localization of GSK-3β and tau (thus facilitating GSK-3β-mediated phosphorylation of tau). Presenilin 1 also binds to other substrates of GSK-3β such as β-catenin, and the amyloid precursor protein (APP), acting as a scaffold for bringing GSK-3β into close proximity with these substrates, which have critical roles in AD and cell survival/apoptotic mechanisms.

2.9 GSK-3β and bipolar disorder

Bipolar disorder (BPD) is characterized by an alternating pattern of emotional highs (mania) and lows (depression), and associated with significant morbidity and mortality, affecting about 1% of the population worldwide. Severe mood alteration affects one's sense of well being, as well as self-esteem, judgment, attention, motivation, learning and memory, sleep, appetite, and overall psychomotor activity. Mood disorders have traditionally been conceptualized as neurochemical disorders of certain neurotransmitter systems such as serotonergic, adrenergic, and glutamatergic and GABAergic systems. However, recent brain imaging and morphometric-postmortem studies demonstrate regional changes in CNS volume, as well as changes in the number and/or size of glia and neurons in discrete brain areas of patients with mood disorders. These data suggest neurotrophic function, cellular growth, death, and resilience as possible factors that

contribute to these disorders (31). Patients respond remarkably well to mood-stabilizing drugs, such as lithium and the anticonvulsant VPA, in spite of their totally different chemical structures. Although the biochemical basis for mood-stabilizer therapies to BPD remains poorly understood, several recent reviews have addressed the possible mechanism of action of lithium and VPA. As lithium is a direct GSK-3β inhibitor, and VPA was reported to inhibit GSK-3β as well, it is an intriguing point that disregulation of GSK-3β may have an influence on this psychiatric disorder.

3. The effects of lithium and VPA on regulating GSK-3β

3.1 Lithium

Lithium has been used for BPD since the late 1940s, and it is an effective treatment for both acute and long-term phases of BPD, as well as adjunctive prophylaxis in unipolar recurrent depression. Side effects of lithium treatment include diabetes insipidus, subclinical hypothyroidism, hyperparathyroidism, and benign neurophilia. Unfortunately, lithium has a narrow therapeutic window and is effective in only 60-80% of BPD patients (32).

Two protein families have been demonstrated as direct cellular targets of lithium and are affected at concentrations used clinically (33). One is the super-family of structurally related phosphomonoesterases, including IMPase, IPP, fructose-1,6-biphosphatase, and the rat PAP phosphatase (RnPIP). Biochemical studies show that the magnesium ion-binding site located in the active site of the enzymes is the target of lithium. Lithium prevents product release and traps products in the active site, resulting in an

uncompetitive inhibition (34). The other target is the family of serine/threonine protein kinases that GSK-3β belongs to. Enzyme kinetic experiments suggest that this inhibition is also through competition for magnesium ion binding. The fact that lithium inhibits both of these two enzyme groups through an interaction with magnesium suggests that this may be a general mechanism of lithium action (33).

Lithium inhibits GSK-3β (Ki = 1 - 2mM) in an uncompetitive mode in vitro (35) and it also inhibits GSK-3β in vivo. Therefore, the various effects of lithium on metabolic, developmental, and neuronal morphology and survival could be explained by its action on GSK-3β.

3.1.1 Molecular changes in lithium treatment

It has been reported that lithium affects neurotransmitter release, metabolism of biogenic monoamines, and neuronal signal transmission through perturbation of the distribution of various cations, including sodium, magnesium, and calcium (32). Lithium can regulate a number of phosphoproteins at the level of expression and/or phosphorylation, including neurofilament proteins (36), microtubule-associated proteins (MAPs) (20), and protein kinase C (PKC) substrates (37).

3.1.2 Neuroprotection of Lithium

Lithium protects neurons from a variety of pro-apoptotic stimuli in vitro and in vivo (32). For example, treatment with lithium for seven days protects cultured rat cerebellar, cerebral cortical, and hippocampal neurons against glutamate-induced excitotoxicity (38). Lithium also protects cultured cerebellar granule neurons against C_2-ceramide (39), β-

amyloid induced neurodegeneration (29), potassium deprivation and serum withdrawal (4). Pretreatment of mice with lithium delays radiation-induced apoptosis in external cerebellar granule cells (40).

Lithium inhibits tau phophorylation through inhibition of GSK-3β, as shown *in vitro* and *in vivo*. Therefore, lithium has been considered as a potential therapy for neurodegenerative diseases in which hyperphosphorylation of tau is a principal pathological feature (32).

3.2 Valproic acid (VPA)

The effectiveness of VPA as an anticonvulsant was discovered serendipitously when other compounds were dissolved in VPA for administration to animals used in experimental models of epilepsy (32). Since then, VPA has been widely used in both generalized and partial epilepsies. VPA was used as a mood stabilizer nearly 35 years ago, and it is effective in acute mania and as prophylaxis for recurrent mania and depression. The side effects of VPA include hepatotoxicity, teratogenicity, and suppression of bone marrow function. Administration of VPA in pregnant women increases the risk of a number of congenital anomalies, such as spina bifida aperta (defects in posterior neural tube closure), congenital heart defects, cleft palate, and limb defects.

3.2.1. Molecular changes in VPA treatment

Although the direct target of VPA has not been identified definitively, it has been reported that VPA increases the level of γ-aminobutyric acid (GABA); inhibits sodium,

potassium, and calcium channel function; inhibits histone deacetylases (HDACs); And, in common with lithium, VPA activates the mitogen-activated protein kinase (MAPK) pathway; increases expression of Bcl-2, and activates AP-1-dependent transcription.

Initially, VPA was reported to directly inhibit GSK-3β activity (41), but this is not supported by a number of further experiments. Some suggested that VPA treatment could lead to an indirect change in GSK-3 activity (42). Others reported that VPA could regulate GSK-3β phosphorylation via activation of protein kinase B (PKB), the upstream enzyme phosphorylating and inactivating GSK-3β (43).

3.2.2. Neuroprotection of VPA

VPA protects cultured rat hippocampal neurons against β-amyloid and glutamate-induced injury (44). VPA also prevents cultured cerebellar granule cells from apoptosis induced by low potassium (3). However, Mora et al. reported that inhibition of PI-3K activity by LY294002 (a specific PI-3K inhibitor) blocks the protection afforded by VPA against apoptosis, whereas lithium can still exert its protection while LY294002 is present. This suggests that VPA protects CGNs against low-K^+-induced apoptosis through PI-3K pathway, while the protection by Li^+ is independent of this pathway (45).

Also, Hall et al. compared the effect of therapeutic concentrations of lithium (1-2 mM) and VPA (1mM) during neuronal maturation. They showed that VPA and lithium induce clustering of synapsin I and induce similar changes in the morphology of axons by increasing growth cone size, spreading, and branching. More importantly, both of them decrease the phosphorylation level of microtubule-associated protein-1B (MAP-1B is a GSK-3β substrate) in developing neurons (42). As *in vitro* kinase assays shows, lithium

partially inhibits GSK-3β activity, while VPA does not inhibit GSK-3β. This finding also supports the idea that both mood stabilizers inhibit GSK-3β activity through different mechanisms: lithium directly inhibits GSK-3β; on the other hand, VPA inhibits GSK-3β indirectly by an as-yet-unknown pathway.

Hypothesis

From the above, we have known that GSK-3β plays a pro-apoptotic role in neuronal cell death, and selective GSK-3β inhibitor such as lithium can protect neurons from many apoptotic insults. In order to understand better how GSK-3β exerts its neurotoxic effect in cell death, and the mechanism of how VPA and lithium regulate GSK-3β activity and promote cell survival, we wish to test the ability of lithium and valproic acid to protect neurons from the pro-apoptotic effects of serum/potassium withdrawal. We planned to use cultured rat cerebellar granule neurons (CGNs) switched to serum-free and low potassium (5mM) medium. The reason for selecting this cell type is that they are abundant and the in vitro culture is homogeneous. Moreover, the death of developing neurons in this model has the classic morphological and biochemical characteristics of apoptosis. The following specific aims are proposed:

1) **Analysis of neuroprotection by lithium and VPA**. This aim is to investigate whether lithium and VPA could prevent trophic-deprivation induced neuronal apoptosis. Briefly, CGNs will be treated with serum free and low potassium medium in absence or presence of lithium or VPA, or other inhibitors of GSK-3β (Indirubin is used as a positive control). After 24h incubation, the neuronal survival will be evaluated by performing MTT assays. If both lithium and VPA are neuroprotective in this model system, the cell viability in lithium- or VPA-treated CGNs is expected to be higher than that in serum-free and low-K^+ treated CGNs.

2) **Mechanism of neuroprotection by lithium or VPA**. In this aim, we will investigate whether protection of CGNs by lithium or VPA involve inhibition of GSK-3β activity by

regulatory phosphorylation at Ser9 of GSK-3β molecules (Aim 2A), and alteration in GSK-3β-specific phosphorylation of tau (Aim 2B). Briefly, CGNs will be treated as outlined in Aim 1 for defined time intervals such as 2h; 6h and immunoblots will be performed using phospho-GSK-3β-specific (Ser9) and phospho-tau-specific (Ser199) antiserum. Here the phosphorylation status of tau will be used as an indicator of GSK-3β activity. If these two drugs could indeed regulate GSK-3β activity, they will either inhibit GSK-3β activation by Ser9 phosphorylation, or reduce GSK-3β enzyme activity.

3) **The effects of lithium or VPA on c-Jun protein expression** In this aim, we will examine whether neuroprotection of CGNs by lithium or VPA involve inhibition of c-Jun protein expression (Aim 3). CGNs will be treated as described in Aim2. Immunoblots will be performed using c-Jun antiserum. If both of the drugs are neuroprotective, we speculate that c-Jun protein expression should be depressed, or at least, should not be elevated.

Materials and Methods

Materials

Basal medium Eagle, heat-inactivated fetal calf serum and gentamicin were purchased from Gibco BRL, Invitrogen Corporation (Grand Island, NY). The culture plates were the products of Sarstedt (Newton, NC) and Corning (Corning, NY). Lithium chloride, valproic acid sodium salt (sodium valproate), indirubin-3'-monoxime, poly-L-lysine, trypsin, DNase, cytosine-β-arabinofuranoside and soybean trypsin inhibitor were from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibodies against phospho-GSKα/β(Ser21/9), phospho-(Ser199)-Tau and c-Jun (N) were obtained from Cell Signaling Technology (Beverly, MA), Sigma-Aldrich and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The mouse anti-GSK-3α/β and the rabbit anti-Tau (H-150) antibodies were from Santa Cruz Biotechnology. The horseradish peroxidase-conjugated donkey anti-rabbit IgG and sheep anti-mouse IgG were from Amersham Pharmacia Biotech (Amersham Place, UK).

Cerebellar granule cell cultures

Primary cultures of cerebellar granule cells were prepared from 7-8-day-old Sprague-Dawley rat cerebella (Charles River) as previously described (46) with some modifications (47). Briefly, cerebelli were dissected and meninges and blood vessels removed. Then cerebelli were minced using a single edged razor blade with two passes at 90° to each other at approximately 0.5 mm intervals. Minced tissue was incubated in 0.25% trypsin solution at 37 °C for 12 min, cells were dispersed by trituration in a

DNase- and soybean trypsin inhibitor-containing solution (0.01% and 0.05%, respectively) and plated onto 24-well plates ($5x10^5$ cells/ml/well), 12-well plates (10^6 cells/2 ml/well) or 6-well plates ($2x10^6$ cells/4 ml/well) coated with 10 μg/ml of poly-L-lysine. Cells were cultured for 6-8 days at 37 °C in an atmosphere of 5% CO_2/95% air in Basal Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 25 mM KCl, 2 mM glutamine, and 100 μg/ml gentamicin (K25+S). Cytosine-β-arabinofuranoside (Ara-C; 10 μM) was added 24 h after plating to limit the number of nonneuronal cells to less than 5%.

Assessment of cell viability

The MTT assay was used to assess the viability of neurons. It has been widely assumed that mitochondrial dehydrogenases in living cells convert soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into an insoluble blue formazan product that can be dissolved in isopropanol and the color intensity measured spectrophotometrically. In this way, MTT assay would assess the integrity of mitochondria characteristic of viable cells. However, recent findings suggest that MTT is taken into cells through endocytosis and reduced primarily in the endosome/lysosome compartment instead of the mitochondria. Nevertheless, the MTT assay, as a measure of cell viability, is still valid because it measures endocytosis, a fundamental feature of most living cells.

The MTT assay was initiated by removing the culture medium and adding MTT (0.3 mg/ml) dissolved in Basal Eagle's medium supplemented with 25 mM KCl, 2 mM glutamine, and 100 μg/ml gentamicin. Following 1 h incubation at 37° C, the medium

was aspirated and 0.3 ml of isopropanol was added to lyse the cells and dissolve the formazan crystals. Aliquots (100 µl) of this solution were pipetted into 96-well microplates and the absorbance was recorded at 570 nm (with background subtraction at 630 nm) in a microplate reader. Cell viability was expressed as a percentage of the absorption in control cultures (=100 %).

Trophic factor withdrawal

Trophic factor withdrawal was carried out on the 6^{th}-7^{th} days in vitro. Cultures were washed twice either with K25-S (Basal Eagle's medium supplemented with 25 mM KCl, 2 mM glutamine, and 100 µg/ml gentamicin) or K5-S (Basal Eagle's medium supplemented with 2 mM glutamine, and 100 µg/ml gentamicin), then the corresponding medium (K25+S or K5-S) was added to the cells for the indicated time periods.

Immunoblotting

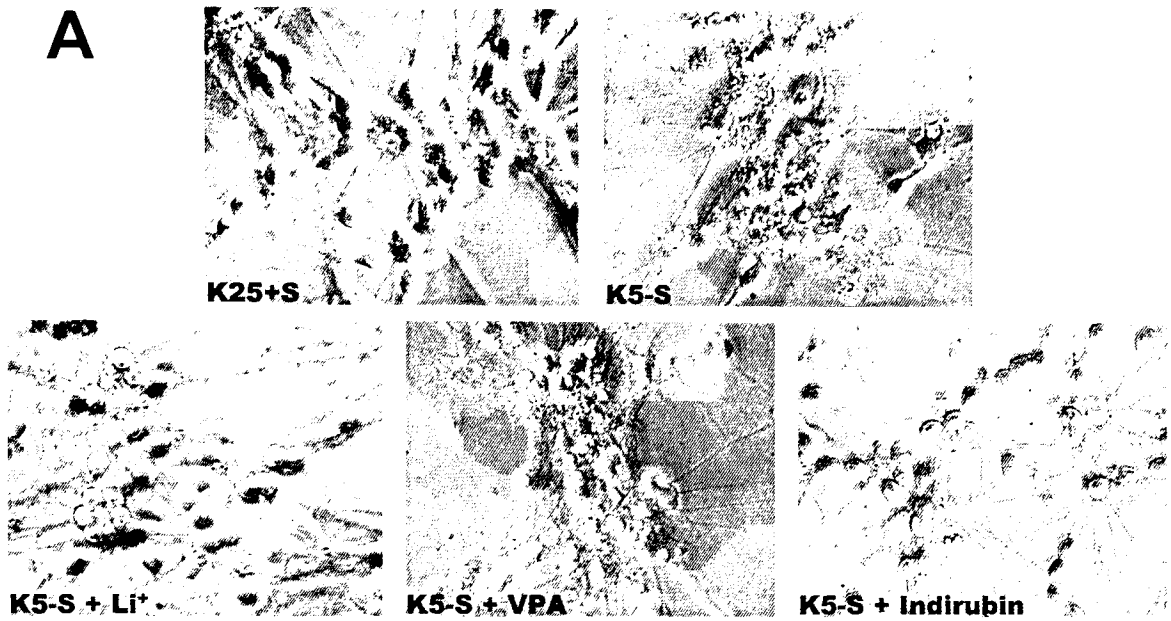
Immunoblot analysis was performed as described previously (47). After removing the culture medium, the cells were lysed in ice-cold lysis buffer (50 mm Tris, pH 7.5, 0.1% Triton X-100, 2 mm EDTA, 2 mm EGTA, 1 µm microcystin, 1 mm Na_3VO_4, 50 mm NaF, 10 mm sodium β-glycerophosphate, 5 mm sodium pyrophosphate, 0.1% (v/v) β-mercaptoethanol and 1 tablet/50 mL of complete protease inhibitor cocktail). After centrifugation at 15,000 *g* for 10 min, cell lysates containing equal amounts of total protein were fractionated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to the Hybond ECL nitrocellulose membrane (Amersham). The filters were blocked for 1 h with 5% skimmed

milk in 1X Tris-buffered saline, 0.5% Tween-20, followed by a 1-h incubation with the primary antibody diluted in the same blocking solution. The antibodies against GSK-3α/β, Tau and c-Jun were used in 1:500 dilution, whereas the anti-phospho-GSK-3α/β (Ser21/9)) and anti-phosphoTau^{199} antibodies were applied at 1:1000 dilution. After having detected the phospho-GSK-3α/β or phospho-Tau^{199} levels, the nitrocellulose membranes were stripped using the Restore Western Blot Stripping Buffer from Pierce, and the corresponding membranes were reprobed for total GSK-3 and total Tau, respectively. Equal loading and uniformity of the protein transfer to the nitrocellulose membrane were also verified by the equal intensity of unspecific bands on the films following the enhanced chemiluminescence reaction.

Results

Lithium, but not VPA can protect cultured CGNs against cell death induced by trophic withdrawal

Primary cultures of cerebellar granule neurons were maintained in medium containing serum (10%) and 25 mM KCl as survival factors. Serum and potassium deprivation triggers GSK-3β-mediated apoptosis in these cultures (4, 9, 48). To investigate the effects of lithium and VPA on neuronal survival in this culture model, on the 6-7th day *in vitro*, serum and potassium were withdrawn (K5-S) in the presence of increasing concentrations of either lithium chloride or valproic acid sodium salt (sodium valproate) for 24 hours. Cell viability was measured by the MTT assay. Consistent with previous findings, more than 60% of the neurons died after 24 hours. As expected, lithium (1-20 mM) provided concentration-dependent neuroprotection (Fig 1B). The specific, direct GSK-3β inhibitor, indirubin-3'-monoxime (indirubin), also protected CGNs against trophic deprivation, providing complete neuroprotection at a concentration of 10 μM. On the contrary, VPA (1-20 mM) was not neuroprotective at all and even potentiated cell death at lower concentrations (see Fig 1B). In addition to the water-soluble sodium valproate, we also treated our cultures with pure valproic acid (dissolved in DMSO), but this preparation was neither neuroprotective (data not shown).



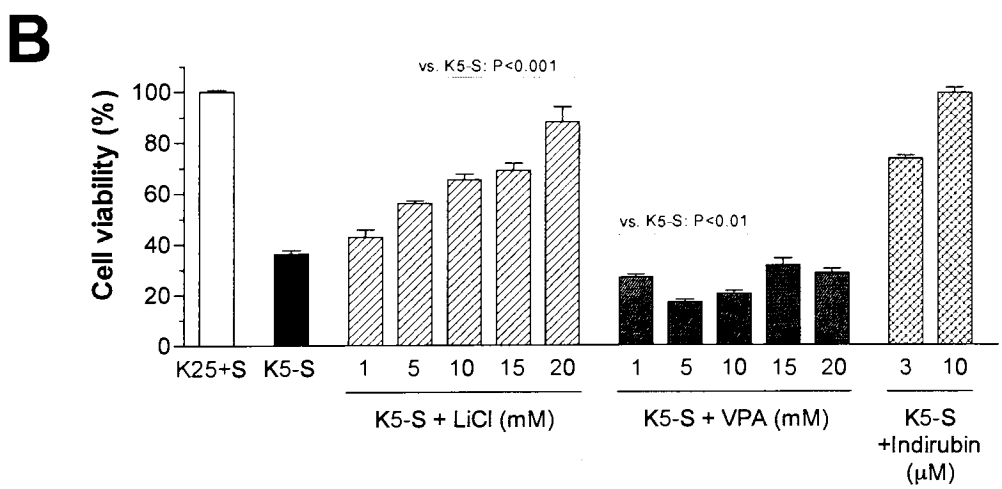


Figure 1A. Microscopic view of cerebellar granule neurons (CGNs) after 24-hour incubation with different chemicals in K5-S medium. **K25+S:** CGNs can be maintained in medium containing serum and 25 mM K^+. **K5-S:** Serum and K^+ deprivation triggers cell death, representing characteristic morphology of apoptosis, including shrinking of the cytoplasm, plasma membrane blebbing, and loss of dendrites. **K5-S + Li:** Lithium can protect CGNs against cell death. **K5-S + VPA:** VPA treatment cannot prevent cell death. **K5-S + Indirubin:** Indirubin can provide complete neuroprotection. Final drug concentrations: [LiCl] = 15 mM, [Indirubin] = 10 μM, [VPA] = 15 mM.

Figure 1B. Lithium, but not VPA can protect cultured CGNs against cell death induced by trophic withdrawal. After 24 hours of serum and potassium withdrawal, more than

60% of the CGNs died. Lithium is protective at therapeutic concentration (1mM) and demonstrates neuroprotection in a dose-dependent manner. Indirubin, a direct GSK-3ß inhibitor, promotes CGN survival significantly. In contrast, VPA cannot prevent CGNs from death, and cell viability is even lower than that in K5-S. Columns and bars represent mean±S.E.M. of 3-6 separate experiments with 3-6 different culture preparations. Statistical significances were determined by one-way ANOVA followed by Bonferroni's test for multiple comparisons.

Lithium, but not VPA, can inhibit GSK-3β activation followed by trophic withdrawal

Trophic withdrawal in cultured CGNs causes the blockade of the PI3-K/PKB survival-signaling pathway leading to GSK-3 activation as a result of decreased inhibitory phosphorylation of GSK-3α and GSK-3β on Ser21 and Ser9, respectively (4, 9, 48, 49). To test whether lithium and VPA can inhibit GSK-3 activation, immunoblot experiments were performed using antibodies against phospho-GSK-3α/β(Ser21/9) and GSK-3α/β to detect the levels of inactive (phosphorylated) GSK-3 and total GSK-3, respectively. Serum and potassium were withdrawn from DIV 6 CGN cultures for 2h or 6 h, in the presence or absence of either 10 mM lithium chloride or 10 mM sodium valproate. The level of phospho-GSK-3, especially of phospho-GSK-3β, significantly decreased after 2 h and remained also low after 6 h, indicative of a rapid and prolonged GSK-3 activation (see Fig 2.). Lithium not only prevented GSK-3 dephosphorylation but even increased the phospho-GSK-3 level above the control (K25+S). Moreover, if lithium was added to the cultures after the first two hours of serum/potassium deprivation, it was able to induce inhibitory phosphorylation of GSK-3 (Fig. 2). These facts imply that lithium, besides inhibiting phosphatases, activates some kinase(s) phosphorylating GSK-3. VPA, however, not only did not inhibit GSK-3 dephosphorylation, but even potentiated it considerably further increasing the proportion of active GSK-3 (see Fig. 2).

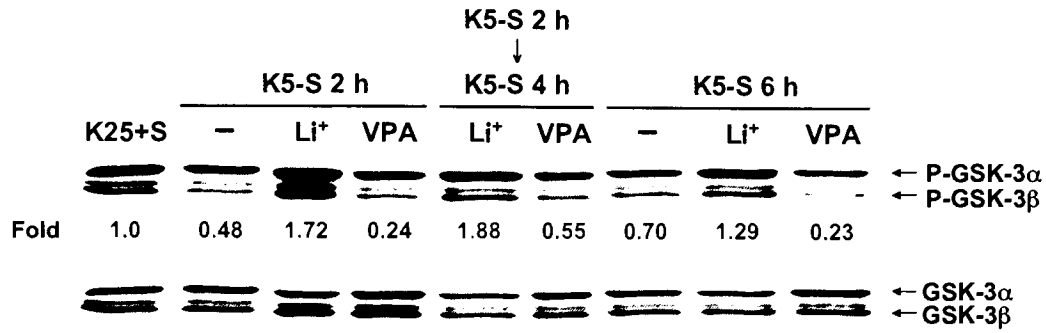


Figure 2. CGN cultures were maintained in medium containing serum (S) and 25 mM KCl (K25+S). Serum/potassium (K5-S) deprivation in the absence or in the presence of LiCl (10 mM), VPA (10 mM) or indirubin (10 μM) was carried out on DIV 6-7, for the indicated time periods. At the end of the treatments, cells were lysed and the cell lysates were immunoblotted with an antibody to phospho-GSK-3α/β(Ser21/9) to detect the level of inactive (phosphorylated) GSK-3. The nitrocellulose membrane was then stripped and reprobed with an antibody to total GSK-3α/β. Phospho-GSK-3β band intensities were determined by densitometry and normalized to the corresponding total GSK-3β band intensities. Fold changes of the normalized phospho-GSK-3β band intensities are represented as ratios to the control normalized phospho-GSK-3β band intensity (K25+S). Immunoblots representative of similar results obtained in 3 separate experiments with 3 different culture preparations are shown.

Lithium, but not VPA, can inhibit GSK-3β-mediated tau phosphorylation

Although, VPA did not prevent the trophic deprivation induced activation of GSK-3β in CGNs, it may affect the enzymatic activity of GSK-3β. The microtublule-associated protein Tau is a well-known in vivo GSK-3β substrate (50-54). GSK-3β has been reported to phosphorylate multiple sites on Tau but these sites can also be phosphorylated by other kinases (24, 55-57). The serine 199 of Tau, however, is exclusively phosphorylated by GSK-3β (24, 51-53). To test if VPA has any effect on GSK-3β enzymatic activity in vivo, we examined Tau phosphorylation on Ser199 following 2 h of serum/potassium withdrawal in DIV 6 CGN cultures. The level of phospho-Ser199-Tau

was detected by immunoblot using a specific anti-phospho-Ser199-Tau antibody. Following serum/potassium withdrawal for 2 hours, Tau became hyperphosphorylated on Ser199 as a biochemical consequence of high GSK-3β activity (see Fig. 3, lane 2). Both lithium and indirubin, which are direct GSK-3β inhibitors, prevented Tau phosphorylation on Ser199 (Fig. 3, lanes 3 and 5). VPA, however, did not inhibit GSK-3β-mediated phosphoryaltion of Tau on Ser199 (Fig. 3, lane 3).

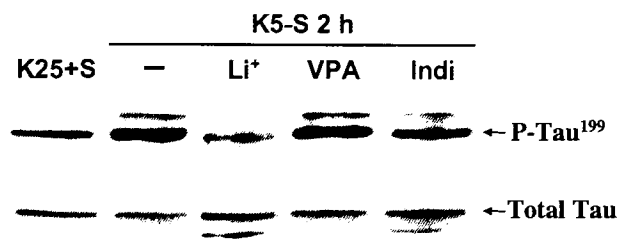


Figure 3. Cerebellar granule neuron cultures were maintained in medium containing serum (S) and 25 mM KCl (K25+S). Serum/potassium (K5-S) deprivation in the absence or in the presence of LiCl (10 mM), VPA (10 mM) or indirubin (10 μM) was carried out on DIV 6-7, for 2 h. At the end of the treatments, cells were lysed and the cell lysates were immunoblotted with antibodies to phospho-Tau^{199} to assess the enzymatic activity of GSK-3β in vivo (serine 199 of Tau is specifically phosphorylated by GSK-3β (24, 51-53). The nitrocellulose membrane was then stripped and reprobed with an antibody to total Tau. Immunoblots representative of similar results obtained in 3 separate experiments with 3 different culture preparations are shown.

Lithium can decrease c-Jun induction, whereas VPA induces c-Jun protein expression

Besides GSK-3 activation, induction of the c-Jun stress response is also essential for apoptosis initiation in trophic deprived CGNs (8, 58). A recent study demonstrated that GSK-3 inhibitors prevent the c-Jun induction following trophic withdrawal in CGNs (9). Therefore, we examined the effects of lithium and VPA on c-Jun expression following

serum/potassium withdrawal. Trophic deprivation induced a massive, prolonged elevation of the c-Jun protein level (Fig. 4, lanes 2 and 6). In accordance with the neuronal survival (see Fig. 1), both lithium and indirubin prevented the high increase in c-Jun expression, whereas VPA further elevated it (Fig. 4).

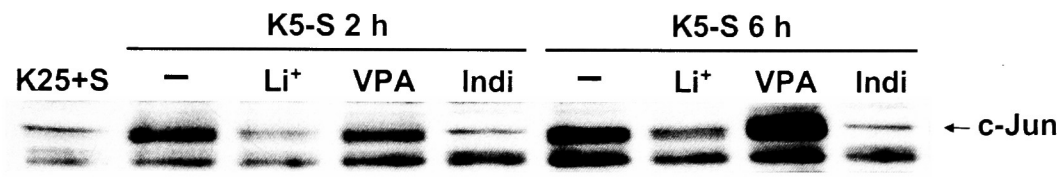


Figure 4. CGN cultures were maintained in medium containing serum (S) and 25 mM KCl (K25+S). Serum/potassium (K5-S) deprivation in the absence or in the presence of LiCl (10 mM), VPA (10 mM) or indirubin (10 μM) was carried out on DIV 6-7, for the indicated time periods. At the end of the treatments, cells were lysed and the cell lysates were immunoblotted with an antibody to c-Jun. An immunoblot representative of similar results obtained in 3 separate experiments with 3 different culture preparations is shown.

Discussion

Valproic acid is a widely prescribed antiepileptic drug and is being used increasingly in the treatment of bipolar disorder, especially in the United States. Like other anticonvulsants, VPA inhibits sodium, potassium and calcium channel function, although its direct in vivo target has not been identified definitely (33). Since the report by Chen et al. (41), describing VPA as a GSK-3 inhibitor in a human neuroblastoma cell line, SH-SY5Y, VPA is regarded as a neuronal GSK inhibitor. This firm belief has been surviving despite the findings by Phiel et al. (59), showing that VPA does not inhibit GSK-3 in Neuro2A cells. This study also demonstrated that the increased level of β-catenin in VPA treated cells, instead of being a result of GSK-3 inhibition (preventing β-catenin phosphorylation and subsequent degradation) is due to histone deacetylase (HDAC) inhibition by VPA. It should be noted that the elevation of β-catenin levels after 1-6 days of VPA treatment in SH-SY5Y cells was the strongest argument for the in vivo GSK-3 inhibitory effect of VPA in the above-mentioned report by Chen et al. (4). Our results in the present study clearly show that, contrary to well-established GSK-3 inhibitors, such as lithium and indirubin, VPA does not inhibit GSK-3 in cerebellar granule neurons, and does not protect against GSK-3-mediated neurotoxicity either. The neuroprotective effect of VPA observed in different experimental paradigms (60-63) is, therefore, most likely the result of its HDAC inhibitory and/or ion channel blocking actions. It was recently shown, e.g., that VPA prolongs the life span of cortical neurons in culture and this neuroprotection is associated with inhibition of HDAC (63a). The same group showed later that in brains of VPA-treated mice 33 genes were up-regulated and 44 genes were

downregulated (63b). These genes could be broadly classified into several functional groups involved in apoptosis, cell growth, cell cycle, cell adhesion, signal transduction, development, protein biosynthesis, receptor activity, metabolism and transport. An upregulated gene of interest was heat shock protein 70 (HSP 70) since HSP 70 has been shown to have a neuroprotective role (64).

Primary cultures of cerebellar granule neurons (CGNs) can be maintained in medium containing serum (10%) and 25 mM KCl as survival factors. The chronic depolarization induced by this high concentration of potassium is thought to imitate endogenous excitatory activity. Serum and potassium deprivation triggers GSK-3-mediated apoptosis in these cultures (4, 9, 48). In agreement with our results, Mora et al. (48) also found that VPA was not protective against combined serum and potassium deprivation in cultured CGNs. In contrast to this, in a study by Li et al. (3), VPA provided neuroprotection in serum/potassium deprived CGN cultures. In this study, however, extremely high cell densities (3-4-fold of the usual ones) were used, and serum and potassium withdrawal for 24 h only resulted in 22% cell death, contrary to the typical 50-70% neuronal death detected in other studies (48, 65-67) including our present report (see Fig. 1B). The higher cell density inevitable means the presence of much more glial cells, making CGN cultures considerably more resistant to trophic deprivation (68). The mechanism how VPA protected against the slight toxicity in those dense CGN cultures (3), therefore, is not clear.

Besides GSK-3 activation, induction of the c-Jun stress response is also essential for apoptosis initiation in trophic deprived CGNs (8, 58). A recent study by Hongisto et al. (9) demonstrated the cooperative action of the c-Jun activator kinase, c-Jun N-terminal

kinase, and GSK-3 in regulating the c-Jun stress response in CGNs. Structurally independent inhibitors of GSK-3, such as lithium, indirubin and FRAT1 (the GSK-3-binding inhibitory protein), prevented the serum/potassium deprivation-induced c-Jun protein increase and subsequent neuronal apoptosis (9). Our results confirm these findings. VPA in our experiments, instead of inhibiting, further potentiated the c-Jun induction, which can give grounds for its deteriorative effect in trophic deprivation. Although, both lithium and VPA have been shown to induce c-Jun expression and to enhance AP-1 mediated gene expression in cell lines and neurons (69-73), only lithium was reported to have an opposite, reducing effect on stress-induced c-Jun responses (9, 75-77).

In summary, our results demonstrate that VPA is not a GSK-3 inhibitor in neurons, and call the attention, that VPA, instead of being neuroprotective, can even exacerbate neuronal death upon trophic deprivation by potentiating the c-Jun stress response.

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